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<p>(21) International Application Number: PCT/GB99/01848</p> <p>(22) International Filing Date: 10 June 1999 (10.06.99)</p> <p>(30) Priority Data:</p> <table border="0"> <tr> <td>9812376.3</td> <td>10 June 1998 (10.06.98)</td> <td>GB</td> </tr> <tr> <td>9814888.5</td> <td>10 July 1998 (10.07.98)</td> <td>GB</td> </tr> </table> <p>(71) Applicant (for all designated States except US): THE QUEEN'S UNIVERSITY OF BELFAST [GB/GB]; 8 Malone Road, Belfast BT9 5BN (GB).</p> <p>(72) Inventors; and</p> <p>(75) Inventors/Applicants (for US only): NELSON, John [GB/GB]; 59 Ashley Avenue, Belfast BT9 7BU (GB). HARRIOTT, Patrick [GB/GB]; 12 William Alex Park, Finaghy, Belfast BT10 0LW (GB). WALLACE, Andrew [GB/GB]; 49 Flat, 2 University Street, Belfast BT7 1FY (GB).</p> <p>(74) Agent: MURGITROYD &amp; COMPANY; 373 Scotland Street, Glasgow G5 8QA (GB).</p>	9812376.3	10 June 1998 (10.06.98)	GB	9814888.5	10 July 1998 (10.07.98)	GB	<p>(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).</p> <p><b>Published</b> Without international search report and to be republished upon receipt of that report.</p>
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(54) Title: PEPTIDE

(57) Abstract

The present invention relates to a new method of delivery of molecules into a cell through the use of a modified signal peptide to which a peptide nucleic acid is linked. The signal peptide will comprise at least one positively charged amino acid residue, or functional equivalent thereof. The addition of such positively charged residues can serve as a linker group for the attachment of peptide nucleic acids to the signal peptide thus increasing the number of peptide nucleic acid sequences delivered by the signal peptide and therefore its functional efficiency. Extension of the signal peptide at the C or N terminus through the addition of a single or multiple charged residue or analogues thereof will modify and improve signal peptide delivery function by increasing the solubility and cell permeability characteristics of the signal peptide.

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1     **"Peptide"**

2

3     The present invention relates to the delivery of  
4     molecules into a cell and the use of modified signal  
5     peptides.

6

7     Specifically, a modified analogue of the signal peptide  
8     sequence from Kaposi syndrome fibroblast growth factor  
9     (kFGF) is used as a cell-permeant vehicle for the  
10    intracellular delivery of covalently linked anti-sense  
11    peptide nucleic acid sequences (PNAs).

12

13    PNAs have potential uses as antisense molecules for the  
14    control of gene expression. Since they are capable of  
15    binding tightly to DNA and RNA targets thus preventing  
16    DNA transcription to RNA and RNA translation to  
17    protein. These molecules thus have two potential uses  
18    of commercial importance:

19

20    1. As research reagents where scientists use  
21        antisense strategies to ablate selected genes in  
22        order to understand their function.

23

24    2. As pharmaceutical compounds for companies seeking  
25        to develop nucleic acid-based therapies.

1 Conventional anti-sense oligonucleotide *in vivo*  
2 delivery is highly inefficient, even if long-lasting,  
3 less polar phosphorothioates are used.  
4

5 This invention covers the use of cell-permeant peptide  
6 delivery systems based on the hydrophobic core  
7 sequences of any signal peptide sequence. A signal  
8 peptide is a short-lived *N*-terminal sequence found only  
9 on nascent proteins which are synthesised in the  
10 endoplasmic reticulum. Signal peptides consist of  
11 three domains:  
12

13 (a) *N*-terminus of 1-5 amino acids, often positively  
14 charged;  
15

16 (b) A hydrophobic core or central region (7-16 amino  
17 acids) which is essential for translocation across  
18 the endoplasmic reticulum membrane; and  
19

20 (c) A more polar *C*-terminal domain (3-7 amino acids)  
21 which is important for specifying the cleavage  
22 site.  
23

---

24 Synthetic peptides consisting of only the hydrophobic  
25 cores are typically insoluble in water. Taking the  
26 signal peptide sequence of Kaposi syndrome-derived FGF  
27 as an example, we have modified these insoluble  
28 sequences by the addition of positively charged amino  
29 acids (for example lysines), which have the effect of  
30 rendering them water soluble without compromising their  
31 ability to translocate across cellular membranes. The  
32 ability to add amino groups in this way allows extra  
33 cargo sequences to be conjugated to these amino groups.  
34

35 It is an object of the present invention to provide a  
36 cell permeable peptide delivery system based on a

1 signal peptide sequence for the intracellular delivery  
2 of peptide nucleic acid sequence.

3

4 According to the present invention there is provided a  
5 cell permeable peptide comprising at least the  
6 hydrophobic core of a signal peptide or an analogue  
7 thereof wherein the peptide is modified by the addition  
8 of at least one positively charged amino acids or  
9 positively charged analogues thereof.

10

11 The signal peptide may be a natural or synthetic signal  
12 peptide or a peptide which is substantially similar  
13 thereto.

14

15 A peptide which is substantially similar to a signal  
16 peptide is at least 60% homologous thereto.

17

18 At least one positively charged amino acid is chosen  
19 from lysine and/or arginine and/or any positively  
20 charged analogues thereof.

21

22 In one particular embodiment the cell permeable peptide  
23 is a modified analogue of Kaposi syndrome fibroblast  
24 growth factor (kFGF).

25

26 The positively charged amino acid consists of one or  
27 more lysine residues.

28

29 The invention further provides the use of cell  
30 permeable peptides as described herein for  
31 intracellular delivery of a molecule.

32

33 Preferably, one or more lysine residues will be  
34 attached to the C terminal of the signal sequence  
35 peptide or signal sequence peptide analogue.

36

1 This positively charged lysine allows the linkage of a  
2 peptide nucleic acid, thus facilitating in vivo  
3 delivery of the said peptide nucleic acid.

4

5

6 The invention also provides a cell permeable peptide  
7 which contains multiple positively charged amino acids  
8 or positively charged analogues thereof wherein a  
9 peptide nucleic acid may be conjugated to each  
10 positively charged residue and wherein the peptide  
11 nucleic acids conjugated by such a means are identical  
12 or different.

13

14 The invention also provides a cell permeable peptide  
15 which comprises at least one positively charged amino  
16 acid residue or functionally equivalent positively  
17 charged analogue thereof conjugated or conjugatable to  
18 a lysine tree, to which multiple peptide nucleic acids  
19 may be joined for transport and presentation.

20

21 The linked peptide nucleic acid sequence may be  
22 antisense.

23

---

24 Preferably, the peptide nucleic acid sequence will be  
25 covalently linked.

26

27 The present invention thus allows the use of cell  
28 permeable peptides as described herein to deliver  
29 peptide nucleic acids to in-vivo targets.

30

31 Use of conventional oligonucleotides is being reduced  
32 due to the development of PNAs (Nielsen, et al., 1991),  
33 which are much more stable, being resistant to enzymic  
34 degradation (Jordan, et al., 1997). PNAs replace the  
35 phosphodiester backbone of nucleic acid with repeating  
36 N-(2-aminoethyl)glycine units to which natural

1 nucleobases are attached through methylenecarbonyl  
2 linkers. Although more stable, PNAs suffer from  
3 similar accessibility problems as phosphorothioates do,  
4 and passive diffusion of unmodified PNA across lipid  
5 membranes is not efficient (Wittung, P., et al., 1995).

6  
7 A small number of native peptide sequences can  
8 translocate across membranes of living cells in an  
9 energy-independent and receptor-independent manner.  
10 These peptides have been used to import active cargo  
11 into the cell. For example a peptide from the  
12 homeodomain of Antennapedia has been successfully used  
13 to import both peptidal inhibitors of protein kinase C  
14 (Theodore, et al., 1995) and conventional anti-sense  
15 oligonucleotides (Allinquant, et al., 1995).

16  
17 The present invention provides use of cell permeable  
18 peptide import (CPPI) to deliver peptide nucleic acids  
19 (PNAs).

20  
21 The present invention provides use of the signal  
22 peptide sequence from Karposi syndrome fibroblast  
23 growth factor (kFGF) for delivery of antisense peptide  
24 nucleic acid sequences (PNAs).

25  
26 The invention provides use of a peptide as defined  
27 herein together with lysine residues for multiple  
28 presentation of peptide nucleic acids.

29  
30 The invention further provides use of peptides as  
31 defined herein together with lysine residues in the  
32 simultaneous presentation of different peptides nucleic  
33 acids.

34  
35 The present invention combines the two above  
36 technologies to use CPPI to deliver PNAs to *in vivo*

1 targets.

2

3 The invention described herein has the following  
4 advantages:

5

6 - The modified signal peptides described in this  
7 invention can be used for the delivery of any  
8 cell-impermeant substance into cells.

9

10 - The signal peptides described in this invention  
11 can be used to improve the delivery of substances  
12 of low permeability into cells.

13

14 - The delivery of substances to particular cellular  
15 sub-compartments can be achieved and improved by  
16 incorporating appropriate targeting peptide  
17 sequences or other modifications to the signal  
18 peptides. Effects are only due to the 'cargo'  
19 substance that they carry. For example, addition  
20 of a myristoyl moiety to the peptide would ensure  
21 that it was preferentially retained at the plasma  
22 membrane.

23

---

24 - The signal peptide delivery system has commercial  
25 value in therapeutic drug-delivery systems  
26 including, but not restricted to, gene therapy,  
27 cancer therapy and anti-infectious agent therapy.

28

29 - This system also has commercial value as a tool  
30 for biochemical and molecular biological research.

31

32 - The modified signal peptides described in this  
33 invention do not, themselves, exhibit any  
34 biological effects nor do they affect cell  
35 viability. Effects are only due to the 'cargo'  
36 substance that they carry.



1 This invention will be exemplified in the following  
2 non-limiting examples with reference to the  
3 accompanying figures wherein:-  
4  
5

6 Figure 1 illustrates carboxyfluorescein labelled kFGF  
7 signal peptide-Lys.Lys.Lys - fluorescence calibration  
8 curve.  
9

10 Figure 2 illustrates carboxfluorescein labelled cell  
11 permeant peptide incorporation by whole human  
12 endothelial cells.  
13

14 Figure 3 depicts incorporation of carboxyfluorescein  
15 labelled signal peptide-Lys.Lys.Lys by cell.  
16

17 Figure 4 illustrates subcellular distribution of  
18 labelled signal peptide in cells.  
19

20 Figure 5 depicts incorporation of labelled kFGF peptide  
21 into human dermal endothelial cells.  
22

23 Figure 6a sets out the signal peptide sequence and  
24 modifications.  
25

26 Figure 6b illustrates simultaneous presentation of 3  
27 PNAs directed to different sites on a target RNA.  
28

29 Figure 6c illustrates multiple presentation of the  
30 single PNA species.  
31

32 Table 1 describes carboxyfluorescein derivatised cell  
33 permeant peptides.  
34

35 Table 2a sets out uptake of cell permeant peptides by  
36 cells.

1 Table 2b sets out cellular uptake of permeant peptides  
2 by BHK cells.

3

4 Table 3 sets out results of washing labelled  
5 antennapedia cells.

6

7 Table 4 sets out washing results for labelled signal  
8 peptide-KKK and cells.

9

#### 10 **EXAMPLE 1**

11

12 This is an example of the intracellular delivery of a  
13 low molecular weight compound (carboxyfluorescein)  
14 which is normally cell impermeant.

15

16 In order to determine the best delivery system, a  
17 comparison of the ability of four different cell  
18 permeant peptides (Table 1) to accumulate in whole  
19 cells was undertaken. The four peptide peptides were  
20 synthesised to contain carboxyfluorescein as a reporter  
21 group (Table 1), allowing intracellular accumulation to  
22 be monitored by fluorescence. Whole cells were exposed  
23 to 50  $\mu$ M solutions of each peptide for 24 hours (37°C)  
24 and accumulation was measured using a fluorometer. The  
25 results of this are shown in Tables 2A and 2B.

26

27 The results shown in the whole column of Table 2A were  
28 provided by cell suspensions being exposed to 50  $\mu$ M  
29 peptide each, for 24 hours at 37°C. Incubations  
30 contained  $3.28 \times 10^6$  cells in 1 ml. Subcellular fractionation  
31 was then carried out. Fluorescence measured with  
32 excitation  $\lambda = 471$  nm, emission  $\lambda = 521$  nm. RFU values  
33 were converted to nMoles per  $10^6$  cells.

34

35 The raw relative fluorescent units (RFU) values were  
36 converted to nMoles per  $10^6$  cells using a calibration

1 curve constructed for each peptide. An example of a  
2 fluorescence calibration curve of fluorescein labelled  
3 kFGF is shown in Figure 1.

4  
5 The kFGF-KKK sequence (see Figure 3) shows similar high  
6 rates of cytosolic and nuclear incorporation compared  
7 with the antennapedia peptide (Table 2A). The PKC and  
8 substance P peptides show much lower incorporation  
9 Table 2A & 2B). Incorporation of the kFGF-KKK sequence  
10 is saturable, as can be seen from the data presented on  
11 Figure 2 and time-dependent as shown in Figure 3.

12  
13 Table 2A shows that antennapedia is lost during  
14 subcellular fractionation. Unlike the antennapedia  
15 peptide, carboxyfluorescein-kFGF signal peptide-KKK is  
16 not loosely attached to the cell surface as shown in  
17 Tables 3 and 4. Unlike the antennapedia peptide,  
18 carboxyfluorescein-kFGF signal peptide-KKK does not  
19 remain membrane-bound as shown by the data presented in  
20 Figure 4.

21  
22 It should be noted from Figure 4 that all cells treated  
23 with carboxyfluorescein - labelled kFGF signal peptide  
24 Lysine-Lysine-Lysine have nuclear and cytoplasmic  
25 incorporation. Unlike antennapedia, very little  
26 remains stuck in the cell membrane.

## 27 28 **EXAMPLE 2 - Anti-sense agents for gene ablation**

29  
30 Conventional oligonucleotide sequences or those in  
31 which the phosphodiester bonds are replaced with  
32 nuclease-resistant bonds (such as the phosphothiorates  
33 and the like) may be conjugated to the kFGF-derived  
34 delivery system for intracellular delivery and  
35 subsequent specific blocking of gene translation or  
36 RNase-targeted destruction of the mRNA in question.

1 Alternatively peptide nucleic acid sequences may be  
2 used, as in example 1.

3  
4 Although the "cargo" to be delivered intracellularly is  
5 referred to in the text and represented in the  
6 accompanying figures as a Peptide Nucleic Acid (PNA),  
7 it should not be limited to such cargo type as the  
8 various configurations of CPPI described in this Patent  
9 could also be used to carry peptide sequences or  
10 oligonucleotide sequences (either native sequences or  
11 modified sequences, such as phosphothiorates).

12  
13 It has been demonstrated that addition of a peptide  
14 nucleic acid sequence does not impede incorporation of  
15 the carboxyfluorescein-kFGF signal peptide-{PNA}-KKK.  
16 The confocal micrograph shown in Figure 5 illustrates  
17 this.

18  
19 **EXAMPLE 3**

20  
21 Nuclear localisation signal (NLS) sequences such as are  
22 found on transcription factors like NF-kappaB may be  
23 conjugated to the kFGF-derived delivery system, as in

---

24 Example 1. Intracellular delivery of NLS peptide  
25 sequences would act as 'bait' to selectively block the  
26 translocation of the selected transcription factor,  
27 thus preventing its action. In this way, genes under  
28 the control of the transcription factor could be  
29 identified on the basis of down regulated expression.

30  
31 **EXAMPLE 4**

32  
33 Signal transduction motifs such as phosphotyrosine-  
34 containing peptide sequences (pYP's) act as docking  
35 sites for a large number of proteins. Such signalling  
36 proteins contain domains that recognise (contextually)

1 the phosphotyrosine residues and bind to them in a  
2 specific manner. pYP's are recognised by SH-2 (Src-  
3 homology-2) domains and PTB (phosphotyrosine binding  
4 domains). Specificity is provided by short amino acid  
5 sequences N-and/or C-terminal of the phosphotyrosine.  
6 Such peptide motifs could be conjugated to the kFGF  
7 peptide-derived delivery system as in Example 1, and  
8 could be used to intracellularly deliver pYP's which  
9 would act as bait, thus allowing signal pathways to be  
10 'interrogated'.

11  
12 The signal sequence of kFGF was modified to contain  
13 three lysines at the C-terminal of the hydrophobic  
14 signal sequence. This procedure is illustrated in  
15 Figure 6A. In this Figure 6A (I) shows the signal  
16 peptide with an attached reporter group. Figure 6A  
17 Part II illustrates the addition of the tri-lysine  
18 extension to the C-terminal of the signal peptide  
19 sequence, thus providing three positive charges which  
20 aid solubility and cell permeability. In Figure 6A  
21 Part IIIB, the peptide nucleic acid forms part of the  
22 linear primary amino acid sequence, with Part IV  
23 illustrating a tri-lysine C-terminal extension to the  
24 peptide nucleic acid sequence providing 3 positive  
25 charges and aiding solubility and cell permeability.

26  
27 Part V of Figure 6A further shows a tri-lysyl extension  
28 at the N-terminal of the signal peptide which provides  
29 3 positive charges aiding solubility and cell  
30 permeability. The addition of the tri-lysyl extension  
31 proximal to the carboxyfluorescein reporter group  
32 enhances its fluorescence. In Vb of Figure 6A, the  
33 peptide nucleic acid sequence initially forms part of  
34 the linear primary amino acid sequence at the N-  
35 terminal of the original peptide, before a tri-lysyl  
36 extension is added to the N-terminal of the peptide

1 nucleic acid extension.

2

3 It should be noted that although the above examples  
4 specifically use the amino acid lysine for the addition  
5 of positive charge, molecules containing similar  
6 properties such as arginine or analogues thereof, of  
7 either of these molecules could also be used.

8

9 This peptide, therefore, can accommodate three PNAs,  
10 each bonded to a lysine epsilon amino group. This can  
11 be extended using the Multiple Antigen Presentation  
12 (MAP) technology to present eight (or more) PNA's on  
13 one kFGF signal sequence. A 'lysine tree' constructed  
14 in this way accommodates eight copies of the same PNA,  
15 thus increasing the effective concentration delivered  
16 by each CPPI.

17

18 An example of the addition of such a lysine tree is  
19 shown in Figure 6C Parts I-IV. In Part I a single  
20 lysine molecule added to the C-terminal of the kFGF  
21 signal peptide sequence allows the multiple PNA lysine  
22 tree to be added to the e-amino group of the lysine  
23 side chain.

24

25 Alternatively, Part II of Figure 6C a lysine molecule  
26 added to the N-terminal of the kFGF signal peptide  
27 sequence allows the multiple PNA lysine tree to be  
28 added to the e-amino group of the lysine side chain.

29

30 Part III of Figure 6C further shows that when a C-  
31 terminal tri-lysine extension is added to the signal  
32 peptide with N-terminal associated multiple PNA lysine  
33 tree, the 3 positive charges aid solubility and cell  
34 permeability of the molecule.

35

36 Part IV of Figure 6C add a tri-lysyl extension at the

1 N-terminal of the signal peptide which is attached to  
2 the lysine group added to allow attachment of the  
3 multiple PNA lysine tree as originally illustrated in  
4 Figure 6C Part II. The addition of the 3 positively  
5 charged molecules at this terminal of the molecule,  
6 proximal to the carboxyfluorescein reporter group  
7 enhances its fluorescence.

8  
9 Alternatively a carrier can be constructed containing  
10 three (or more) different PNAs directed towards  
11 different sites on the same target mRNA. This strategy  
12 has been termed 'molecular triangulation' (Branch,  
13 A.D., 1998).

14  
15 Figure 6B illustrates this process of 'molecular  
16 triangulation'. Figure 6B Part I shows the signal  
17 peptide with a C-terminal tri-lysyl extension which  
18 allows three different PNA sequences to be conjugated  
19 to the epsilon-amino groups of the three lysines.

20  
21 Figure 6B Part III shows the addition of a further  
22 three lysines to the molecule of Part I, which adds  
23 three positive charges, which aid solubility and cell  
24 permeability. Figure 6B Part III shows the addition of  
25 the tri-lysyl extension to the N-terminal of the  
26 molecule of Part I. Again the 3 positive charges aid  
27 the solubility and cell permeability of the molecule,  
28 which their proximal location to the carboxyfluorescein  
29 reporter group enhances its fluorescence.

30  
31 Figure 6B, Part IV, illustrates an N-terminal tri-lysyl  
32 extension added to the kFGF signal peptide sequence,  
33 which subsequently allows three different PNA sequences  
34 to be conjugated to the epsilon-amino groups of the  
35 lysines.

36

1 Further, this molecule has 3 lysines added at the C-  
2 terminal to add positive charge which aid solubility  
3 and cell permeability. Figure 6B Part V shows the  
4 signal peptide again with the three peptide nucleic  
5 acid associated tri-lysine extension at the N-terminal,  
6 but with the addition of the further 3 lysine groups  
7 also being made to the N-terminal where they will have  
8 the effect of aiding solubility and cell permeability,  
9 which also enhance the fluorescence of the  
10 carboxyfluorescein reporter group due to their  
11 proximity.

12  
13 Further to the sequences illustrated in Figures 6A and  
14 6C additional tri-lysine extensions at either end of  
15 the molecule, appears to aid solubility and cell  
16 permeability to allow PNA sequences to be transported.  
17 Therefore in addition to using lysine residues to  
18 attach to PNA sequences, additional tri-lysine  
19 extension is recommended. Examples of presentation  
20 peptide using the additional try-lysine is demonstrated  
21 in Figures 6B (II-IV), Figures 6C (III-IV) and Figures  
22 6A (IV, IVb, V, Vb).

---

23  
24 Lysine extensions comprising more or less than three  
25 lysine residues may also be useful to provide  
26 additional solubility and cell permeability.

27  
28 The lysine extension may be provided next to a  
29 carboxyfluorescein reporter group to enhance its  
30 fluorescence.

31



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1     CLAIMS

2

3     1     A cell permeable peptide comprising at least the  
4           hydrophobic core of a signal peptide or an  
5           analogue thereof wherein the peptide is modified  
6           by at least the addition of at least one  
7           positively charged amino acid or positively  
8           charged analogue thereof.

9

10    2     A cell permeable peptide as claimed in claim 1  
11           wherein the signal peptide is a natural or  
12           synthetic signal peptide or a peptide which is  
13           substantially similar thereto.

14

15    3     A cell permeable peptide as claimed in claim 1 and  
16           2 wherein at least one positively charged amino  
17           acid is chosen from lysine and/or arginine and/or  
18           any positively charged analogue thereof.

19

20    4     A cell permeable peptide as claimed in any  
21           preceding claim wherein the cell permeable peptide  
22           is a modified analogue of Kaposi syndrome  
23           fibroblast growth factor (kFGF).

24

25    5     A cell permeable peptide as claimed in any  
26           preceding claim where in the positively charged  
27           amino acid consists of one or more lysine  
28           residues.

29

30    6     A cell permeable peptide as claimed in claim 5  
31           wherein one or more lysine residues are attached  
32           to the C-terminal of the signal sequence peptide  
33           or signal sequence peptide analogue.

34

35    7     A cell permeable peptide as claimed in any of  
36           claims 1 to 6 which contains multiple positively

1 charged amino acids or positively charged  
2 analogues thereof, wherein a peptide nucleic acid  
3 may be conjugated to each positively charged  
4 residue and wherein the peptide nucleic acids  
5 conjugated by such means are identical or  
6 different.

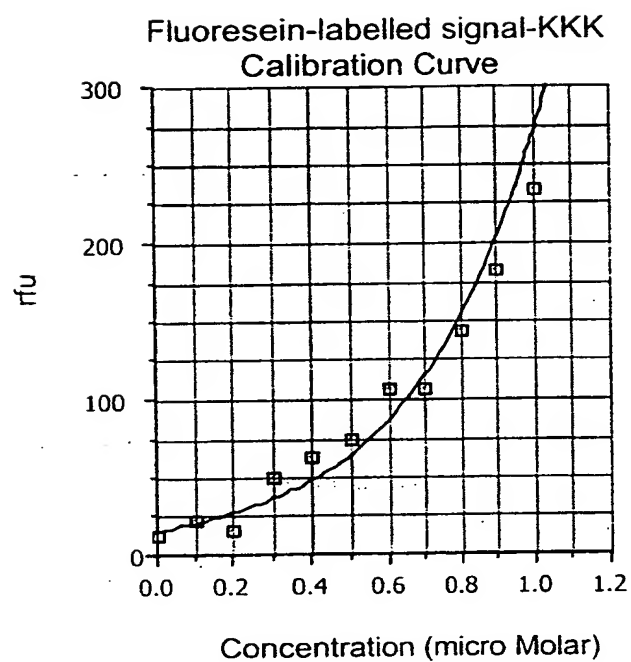
7  
8 8 A cell permeable peptide as claimed in any of  
9 claims 1 to 6 which comprises at least one  
10 positively charged amino acid residue or  
11 functionally equivalent positively charged  
12 analogue thereof, conjugated or conjugatable to a  
13 lysine tree, to which multiple peptide nucleic  
14 acids may be joined for transport and presentation  
15 of multiple peptide nucleic acids.

16  
17 9 Use of cell permeable peptides claimed in any of  
18 the preceding claims for intracellular delivery of  
19 a molecule.

20  
21 10 Use of a cell permeable peptide as claimed in any  
22 of claims 1 to 8 to deliver peptide nucleic acids  
23 to *in-vivo* targets.

24

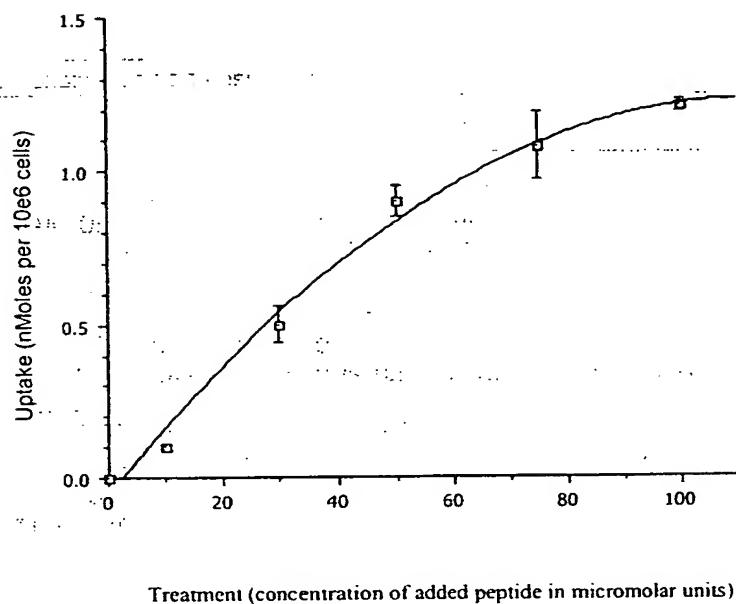
Figure 1



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Figure 2

Whole cell uptake of kFGF-KKK by SK-HEP1 cells (human endothelial)



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Figure 3



15 minutes



30 minutes



45 minutes



1 hour



4 hours



24 hours

Figure 4

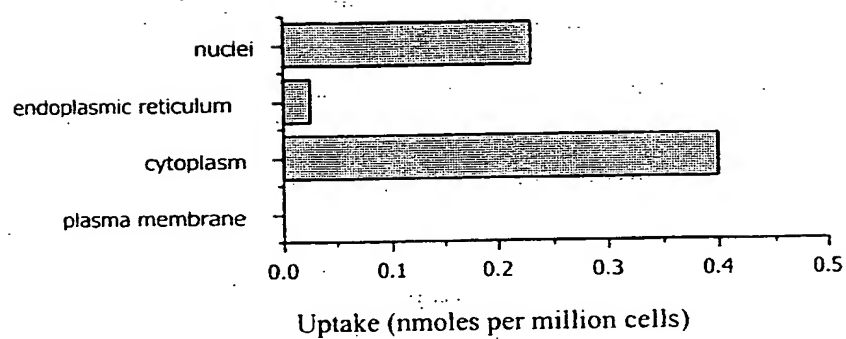


Figure 5

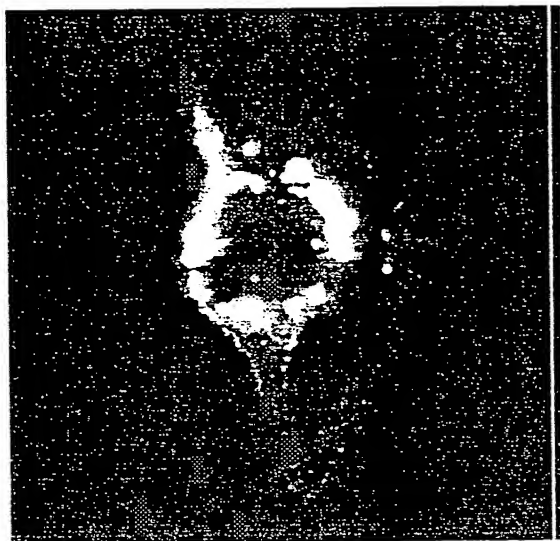




Figure 6A

A.A.V.A.L.L.P.A.V.L.L.A.L.L.A.P

6A(I).

CarboxyFluor -A.A.V.A.L.L.P.A.V.L.L.A.L.L.A.P

6A(II)

CarboxyFluor -A.A.V.A.L.L.P.A.V.L.L.A.L.L.A.P. K.K.K

6A(III)

CarboxyFluor -A.A.V.A.L.L.P.A.V.L.L.A.L.L.A.P-PNA SEQUENCE

6A(IIIb)

CarboxyFluor -A.A.V.A.L.L.P.A.V.L.L.A.L.L.A.P-PNA SEQUENCE-K.K.K

6A(IV)

CarboxyFluor -A.A.V.A.L.L.P.A.V.L.L.A.L.L.A.P. K.K.K-PNA SEQUENCE

6A(IVb)

CarboxyFluor -K.K.K-A.A.V.A.L.L.P.A.V.L.L.A.L.L.A.P-PNA SEQUENCE

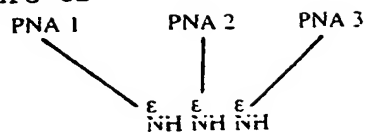
6A(V)

CarboxyFluor -K.K.K-PNA SEQUENCE-A.A.V.A.L.L.P.A.V.L.L.A.L.L.A.P

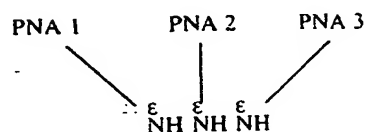
6A(Vb)

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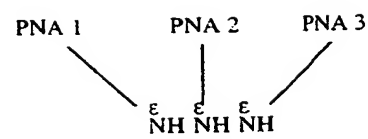
Figure 6B

6B(I)

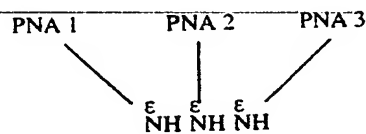
CarboxyFluor-A.A.V.A.L.L.P.A.V.L.L.A.L.L.A.P. K--K

6B(II)

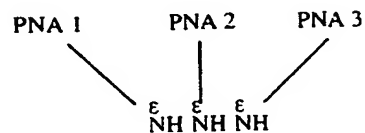
CarboxyFluor-A.A.V.A.L.L.P.A.V.L.L.A.L.L.A.P. K--K-K.K.K.K.

6B(III)

CarboxyFluor-K.K.K. A.A.V.A.L.L.P.A.V.L.L.A.L.L.A.P. K--K-K

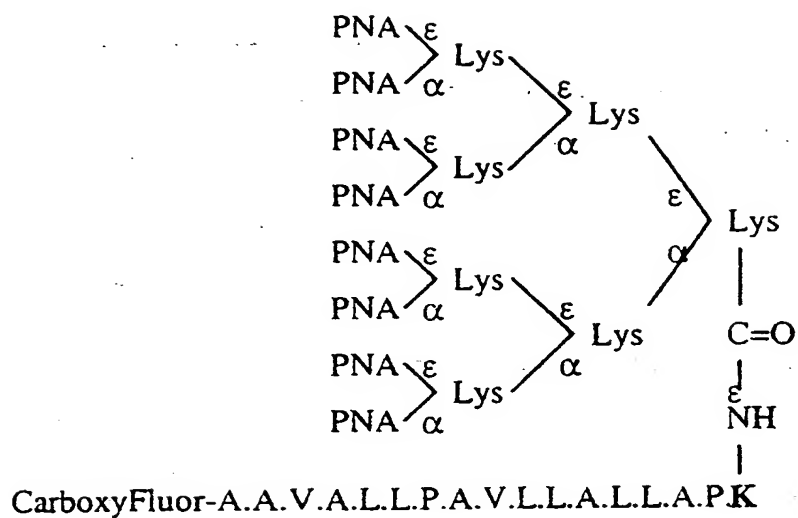
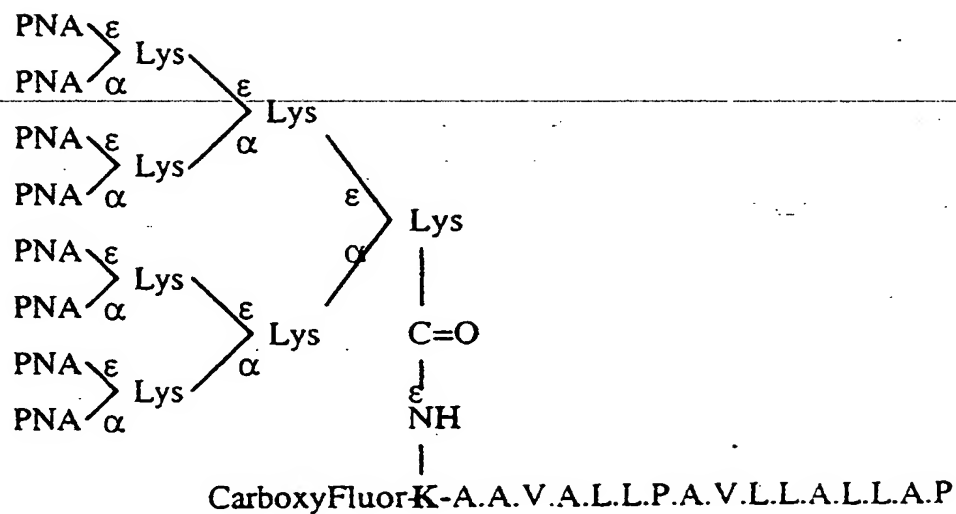
6B(IV)

CarboxyFluor-K--K-K. A.A.V.A.L.L.P.A.V.L.L.A.L.L.A.P. K.K.K

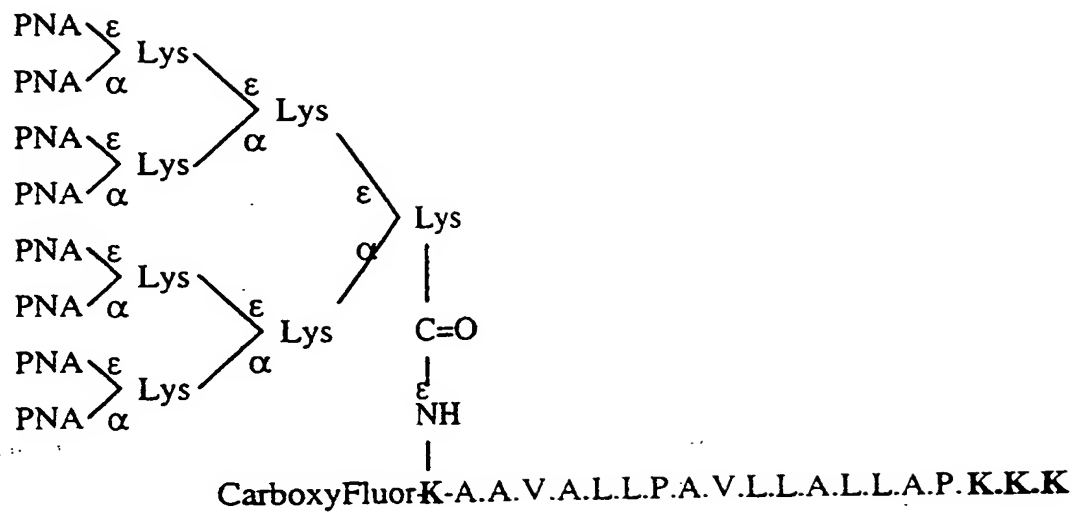
6B(V)

CarboxyFluor.K.K.K -K--K--K. A.A.V.A.L.L.P.A.V.L.L.A.L.L.A.P

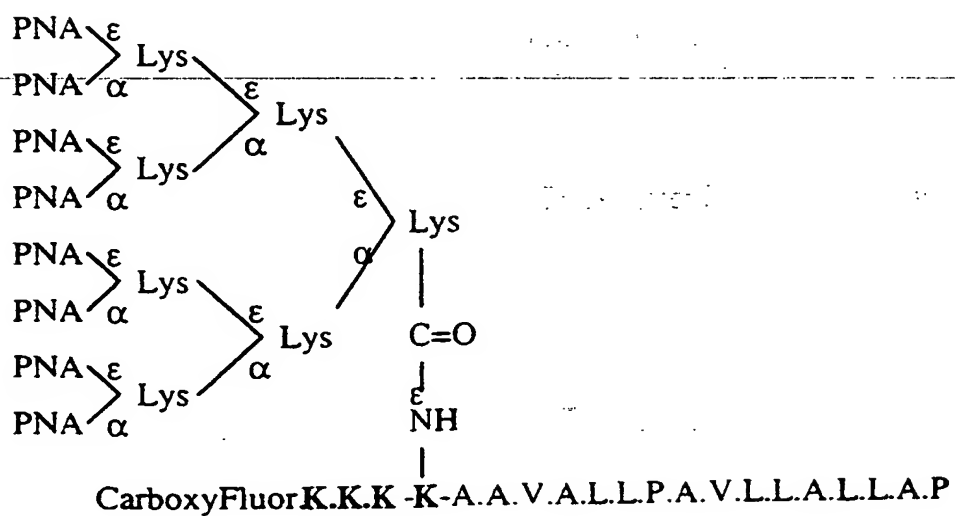
FIGURE 6C

6C(I)6C(II)

6C(III)



6C(IV)



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Table 1

Carboxyfluorescein-derivatised Cell Permeant Peptides*																				
kFGF signal sequence	cFl	A	A	V	A	L	L	P	A	V	L	L	A	L	L	A	P	K	K	K
PKC Pseudo - substrate	cFl	R	F	A	R	K	G	A	L	R	Q	K	N	V	H	E	V	K	N	
Substance P	cFl	R	P	R	P	Q	Q	F	Ø	G	L	M								
Antennapedia	cFl	R	Q	I	K	I	W	F	Q	N	R	R	M	K	W	K	K			

\*Modifications of original sequence marked in bold (Ø = ornithine, cFl = carboxyfluorescein).

Table 2A

	*WHOLE CELL nmoles per 10 <sup>6</sup> cells	CYTOSOL nmoles per 10 <sup>6</sup> cells	NUCLEI nmoles per 10 <sup>6</sup> cells
FGF-KKK	0.79	0.37	0.35
KKK-FGF-KKK	0.24	0.046	0.15
Substance P	0.03	0.005	0.015
PKC pseudo - substrate	0.034	0.015	0.007
Antennapedia	1.22	0.34	0.35

\*Cell suspensions were exposed to 50  $\mu$ M peptide each, for 24 hours, at 37°C,  $\lambda$  = 471nm, emission  $\lambda$  = 521nm. RFU values were converted to nMoles per 10<sup>6</sup> cells

Table 2B

CPPI sequence tested	Amount in nuclei (nmoles per 10 <sup>6</sup> cells)	Amount in cytosol (nmoles per 10 <sup>6</sup> cells)	Cytosolic concentration ( $\mu$ M)
kFGF signal peptide	0.035	0.0567	13.5
SubstanceP analogue	0.0005	0.0018	0.42
PKC pseudosubstrate	0.0005	0.00156	0.37

Table 3

Treatment	rfu
1st PBS wash -	114
2nd PBS	57.34
3rd	21.08
4th PBS/acid wash	15.36

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Table 4

Incorporation Treatment	incorporation (nmoles per 10 <sup>6</sup> cells
PBS wash (after 15min exposure)	0.64
Acid Wash (15min)	0.525
PBS wash (after 24hour exposure)	0.75
Acid wash (after 24hour exposure)	0.53

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C12N 15/87, A61K 31/70
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(54) Title: CELL-PERMEABLE PEPTIDE

(57) Abstract: The present invention relates to a new method of delivery of molecules into a cell through the use of a modified signal peptide to which a peptide nucleic acid is linked. The signal peptide will comprise at least one positively charged amino acid residue, or functional equivalent thereof. The addition of such positively charged residues can serve as a linker group for the attachment of peptide nucleic acids to the signal peptide thus increasing the number of peptide nucleic acid sequences delivered by the signal peptide and therefore its functional efficiency. Extension of the signal peptide at the C or N terminus through the addition of a single or multiple charged residue or analogues thereof will modify and improve signal peptide delivery function by increasing the solubility and cell permeability characteristics of the signal peptide.

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## INTERNATIONAL SEARCH REPORT

International Application No

PCT/GB 99/01848

A. CLASSIFICATION OF SUBJECT MATTER  
 IPC 6 C07K14/50 C12N15/87 A61K31/70

According to International Patent Classification (IPC) or to both national classification and IPC

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Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 97 04006 A (HERRMANN FRIEDHELM.; BRACH MARION (DE); KIEHNTOFF MICHAEL (DE)) 6 February 1997 (1997-02-06) the whole document	1-3, 5-7, 9, 10
A	DU C ET AL., : "Conformational and topological requirements of cell-permeable peptide function" JOURNAL OF PEPTIDE RESEARCH, vol. 51 (3), March 1998 (1998-03), page 235-243 XP000856550 the whole document --- -/--	1-10



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A	<p>ALLINQUANT B ET AL., : "Downregulation of amyloid precursor protein inhibits neurite outgrowth in vitro"</p> <p>THE JOURNAL OF CELL BIOLOGY, vol. 128, no. 5, March 1995 (1995-03), pages 919-927, XP000856389 cited in the application abstract page 920, left-hand column, paragraph 3 page 923, right-hand column, paragraph 2 -page 925, right-hand column, last paragraph</p>	1,9,10
A	<p>LIN Y-Z ET AL., : "Inhibition of nuclear translocation of transcription factor NF-kappaB by a synthetic peptide containing a cell membrane-permeable motif and nuclear localization sequence"</p> <p>JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 270, no. 24, 1995, pages 14255-14258, XP002050723 the whole document</p>	1,2,9
A	<p>FULLER-PACE F ET AL., : "Cell transformation by kappaFGF requires secretion but not glycosylation"</p> <p>J. CELL. BIOL., vol. 115, no. 2, 1991, pages 547-555, XP000856406 page 547 -page 548</p>	4
A	<p>ROJAS M. ET AL., : "Controlling epidermal growth factor (EGF)-stimulated Ras activation in intact cells by a cell-permeable peptide mimicking phosphorylated EGF receptor"</p> <p>JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 271, no. 44, 1996, page 27456-27461 XP002124151 the whole document</p>	1,2,9

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International Application No  
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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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